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Isolation of Viridicatin from *Penicillium palitans*

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Summary. Viridicatin, $C_{15}H_{11}O_2N$, has been shown to be a metabolic product of several fungi including *P. palitans*, *P. olivino-viride*, *P. puberulum*, *P. martensii*, *P. crustosum*, *P. granulatum*, and *P. cyclopium*. Although these strains also produce the tremorgenic mycotoxin, tremortin, no toxicity could be shown for viridicatin. Viridicatin does not appear to be a component of the tremortin molecule. A quantitative colorimetric assay has been developed for viridicatin.

It was previously reported that a strain of *Penicillium palitans* NRRL 3468, isolated from moldy commercial feed that may have been involved in the deaths of several dairy cows, produced a tremorgenic toxin (Ciegler, 1969). We have since shown that this strain actually produces three toxins which have tremorgenic properties—tremortins A, B, and C (Hou and Ciegler, 1970). However, these three toxins did not appear to account completely for the toxic properties of crude chloroform extracts of *P. palitans*. Mice that had been injected with pure tremorgens either trembled violently, convulsed, and then died or trembled, recovered, and showed no further symptoms. Mice that recovered from the trembling process after injection with crude extracts, however, often died quietly after a period of apparent continuing intoxication in which the only overt symptoms were ruffled fur, general malaise, and anorexia; the eyes were often closed by death and covered with mucoid material. We subsequently attempted to isolate and identify a potential additional toxic substance from the mold mycelium. This paper reports the isolation of viridicatin (Cunningham and Freeman, 1953; Bracken *et al.*, 1954) from the mycelium of *P. palitans*.

Material and Methods

Cultures and Fermentation. The strain of *P. palitans* NRRL 3468 originally isolated from moldy commercial feed was principally used in this investigation.

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Additional *Penicillium* species came from the ARS Culture Collection at our Laboratory. All cultures were maintained on Difco malt-yeast extract agar slants.

For purposes of fermentation, the spores from a single 1- to 2-week-old slant were suspended in 10 ml of sterile distilled water and used to inoculate Fernbach flasks each containing 500 ml of Czapek Dox broth supplemented with 0.5% yeast extract. Corn steep liquor (2%) was later substituted for yeast extract. Incubation was static at 28° C for 10 to 12 days. At time of harvest the pH of the supernate was 8.2.

Mycelium Extraction. After recovery by filtration through cheesecloth, the mycelium was squeezed dry and then homogenized with chloroform:methanol (70:30 v/v) in a gallon-sized Waring Blendor until the cup became quite warm; two extractions were made, the second using only chloroform, and the extracts combined. Extracts were filtered through a large Büchner funnel and the solids discarded. Water was removed by anhydrous sodium sulfate and the extract taken to dryness at 45—50° C in a rotary flash evaporator. The residual solids were redissolved in the minimal volume of chloroform required and chromatographed on a silica gel (70—325 mesh) column; chloroform was used for development. After the tremortin fraction was eluted, as determined by thinlayer chromatography (TLC), a second fraction was washed from the column with chloroform:methanol (95:5 v/v). Collection tubes containing this latter fraction were pooled and the solvent was removed by flash evaporation. Residual solids were put into solution with boiling chloroform, and the hot solvent was filtered to remove insolubles. On cooling, colorless needle-like crystals precipitated that were then recrystallized from the same solvent. Additional crystals were recovered after the remaining solution was refrigerated at —20° C overnight. The product was washed with cold chloroform, air-dried, and sublimed under vacuum at 205° C.

Synthesis. Viridicatin was synthesized by the method of Bracken *et al.* (1954) although the overall yield was only about 30%. Admixture of synthesized viridicatin with the substance isolated from *P. palitans* did not depress the melting point.

Analysis. Viridicatin was detected by TLC on Silica Gel G-HR plates developed with chloroform:acetone (93:7 v/v) and sprayed with 1% $FeCl_3$ in butanol. At an approximate R_f of 0.20, viridicatin quickly turned greenish blue but became black upon heating a few minutes at 50—60° C (Fig. 1).

To quantitate, a specific volume of extract was spotted on the TLC plate and, after development, the zone containing viridicatin was scraped off and collected in a small test tube. One and a half milliliters of reagent (4—5 mg $FeCl_3$/10 ml methanol) was added to the tube and the contents were agitated with a vortex mixer. The tube was centrifuged and the color intensity of the clear supernatant solution determined at 650 mμ in a Beckman DB spectrophotometer; Beer's law was followed between 30 and 120 μg viridicatin. The presence of the tremortins did not interfere with the test (Fig. 2).

The crude chloroform:methanol extract of the mycelium could also be analyzed directly for viridicatin by adding 1 ml of color reagent to 1 ml of extract, but the precision was not so good as in the preceding method.

Results and Discussion

The compound following the tremorgens on elution from the column was identified as viridicatin (Fig. 3) based on the following criteria: Elemental analysis, C, 76.06%; H, 4.86%; N, 5.79%; oxygen by difference, 13.29% (theoretical, C, 75.93; H, 4.68; N, 5.90; O, 13.49). The

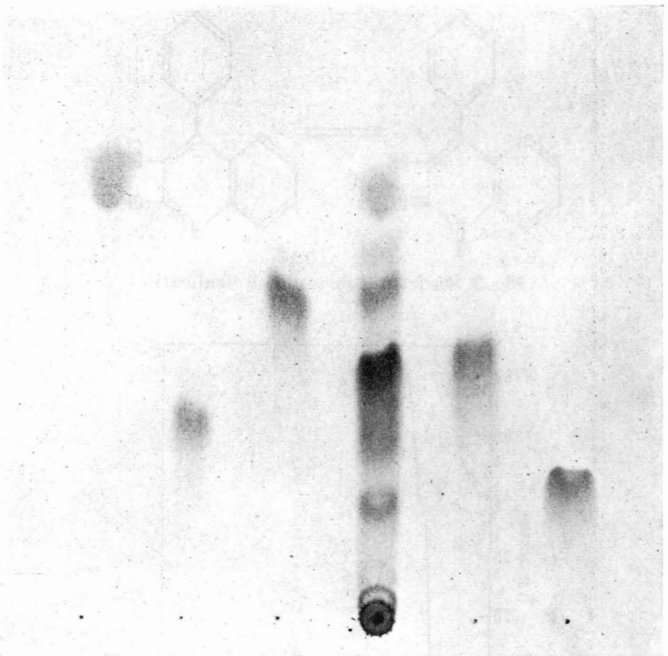

Fig. 1. Thin-layer chromatogram *TLC* of a chloroform-methanol extract of *Penicillium palitans* mycelium. TLC on Silica Gel G-HR; solvent, chloroform:acetone (93:7, v/v); indicator, 1% $FeCl_3$ in butanol. *1* Ergosterol; *2* tremortin C; *3* temortin B; *4* crude solvent extract of *P. palitans* mycelium; *5* tremortin A; and *6* viridicatin

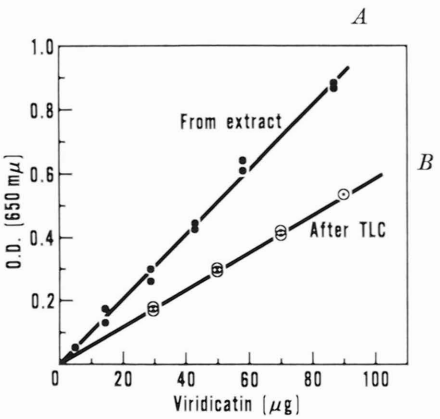

Fig. 2. Colorimetric analyses for viridicatin. *A* Direct method: 1 ml of color reagent plus 1 ml of solvent extract of mycelia; and *B* 1.5 ml of color reagent plus viridicatin zone from TLC

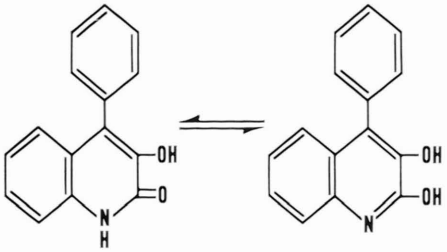

Fig. 3. Structural formula of viridicatin

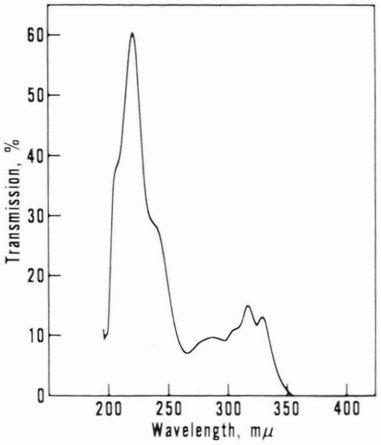

Fig. 4. Ultraviolet spectrum of viridicatin in methanol

elemental analysis and molecular formula, $C_{15}H_{11}O_2N$, were confirmed by high-resolution mass spectroscopy, which also gave an m/e, 237.08. The melting point, 262.9 to 263.5° C (determined with a Mettler FP-1 apparatus) was not depressed on admixture with authentic viridicatin produced either by synthesis or from *P. cyclopium*. The compound cochromatographed with authentic viridicatin even with several solvent systems and gave a blue to gray-blue color on reaction with $FeCl_3$. Ultraviolet (UV), infrared, and mass spectra of viridicatin are reproduced in Figs. 4—6 since previously published spectra are poor and difficult to evaluate. In the UV, there are peaks (in methanol) at 330 (ε, 15×10^4), 317 (ε, 14×10^4), 285 (ε, 17×10^4), 221.5 (ε, 37×10^3), and shoulders at 307 and 238 mμ.

Production. Approximately 350 mg viridicatin was isolated from 270 g dry myelin collected from 45 Fernbach flasks (22.5 l CzapekDox plus 0.5% yeast extract medium) after 10 days of static fermentation.

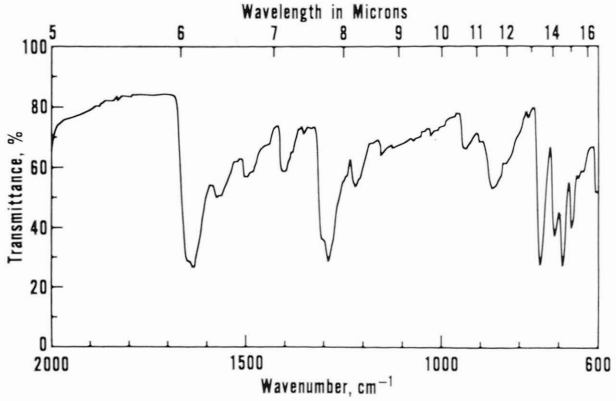

Fig. 5. Infrared spectrum of viridicatin. Recorded from a coated KRS-5 plate with a Beckman IR-8 infrared spectrophotometer

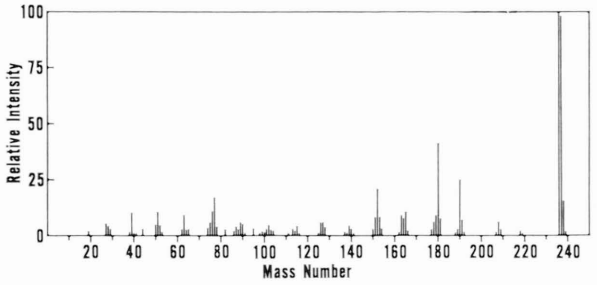

Fig. 6. Mass spectrum of viridicatin from *P. palitans*. Recorded with a Nuclide mass spectrometer

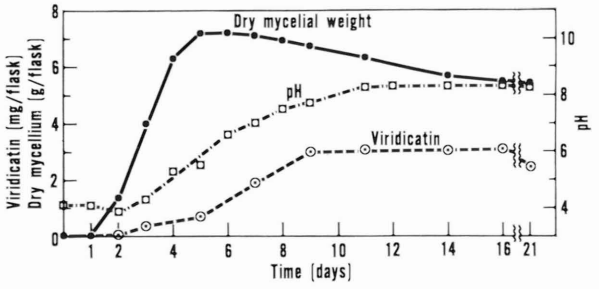

Fig. 7. Production of viridicatin by *P. palitans*. Medium: Czapek-Dox plus 2% corn steep liquor, 500 ml per Fernbach flask. Incubated statically at 25° C

The data in Fig. 7 obtained from the use of Czapek-Dox plus 2% corn steep liquor show that most production occurred after maximum growth was attained and then peaked at about 9 days. No production was detected in shaken flask culture although growth was good.

In a survey of the genus *Penicillium*, we found viridicatin was produced by various strains of the following species all located in the subsection Fasciculata, section Asymetrica: *P. olivino-viride* Biourge, *P. puberulum* Bainier, *P. martensii* Biourge, *P. crustosum* Thom, *P. granulatum* Bainier, and *P. cyclopium* Westling. Only *P. cyclopium* and *P. viridicatum* have previously been reported to produce viridicatin (Cunningham and Freeman, 1953; Bracken *et al.*, 1954). Of more interest was our observation that viridicatin was always found in conjunction with the tremortins; the converse was also true.

Curiously, the molecular weight of viridicatin (237) plus that of ergosterol (396.63), which these molds produce in considerable quantity (Fig. 1), is a little more than the molecular weight, of tremortin A (633), as determined by mass spectroscopy. In addition, finding of viridicatin always with the tremortin plus the occurrence of peaks in the UV, found both in tremortin and in ergosterol (295, 283, 273 mμ), as well as a positive Liebermann-Burchard reaction for the tremortin, led us to conjecture that the tremortin was a composite of both viridicatin and ergosterol. However, we have not been able to demonstrate either ergosterol or viridicatin as a component of tremortin A after treating it with mild and strong acid or base. Nor does mass spectroscopy indicate that viridicatin is a component. The structures of the tremortins have yet to be determined.

Initial experiments indicated that an intraperitoneal (i.p.) injection of 15.25 mg viridicatin/kg mice was lethal within 48 hr. However, in subsequent experiments, we were unable to demonstrate toxicity at doses even four times higher. Nor did these high dosage levels of viridicatin show any synergistic activity when injected (i.p.) with sublethal doses of tremortin A (0.5 mg/kg). Hence, viridicatin does not appear to be the compound involved in the toxicity (other than that caused by the tremortins) shown by crude extracts from *P. palitans*. Our investigation in this area is continuing.

A screen for antibiotic activity confirmed literature reports that viridicatin is comparatively inactive (Cunningham and Freeman, 1953). The compound also demonstrated no toxicity towards the tissue culture lines, Eagle's KB cells and Earle's L cells (Perlman *et al.*, 1969).

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